REMARKS

In the communication from the Examiner, the Examiner requires that Applicants refer to a specific amino acid sequence. Applicants respectfully traverse this requirement for the reasons discussed below.

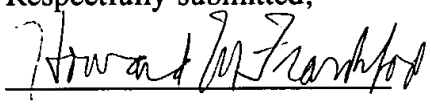
The specific amino acid residues provided in claims 5-7, 11 and 18 correspond to the residues of Domain 4 of the beta chain of GM-CSF/IL-3/IL-5 ($D4\beta_c$) cytokine receptors. Even though the residues of $D4\beta_c$ are not fully disclosed in the specification with a deposit accession number, applicants respectfully submit that the $D4\beta_c$ sequences are known and readily available to the public. M.P.E.P. § 2404.01 states that:

> "In an application where the invention required access to specific biological material, an applicant could show that the biological material is accessible because it is known and readily available to the public. The concepts of "known and readily available" are considered to reflect a level of public accessibility to a necessary component of an invention disclosure that is consistent with an ability to make and use the invention. To avoid the need for a deposit on this basis, the biological material must be known and readily available-neither concept alone is sufficient."

The "biological material" in this case is the $D4\beta_c$ sequences (residues 338-438) of GM-CSF/IL-3/IL-5 receptors. These sequences are disclosed and/or referred to in many scientific publications, *i.e.,* Hayashida K., et al., *Proc. Natl. Acad. Sci. USA*, 87, 9655-9659 (1990); Lock P. et al., *Proc. Natl. Acad. Sci. USA*, 91, 252-256 (1994), submitted herewith as Exhibit A and Exhibit B, respectively. Since these sequences have been disclosed in many publications for at least 13 years, Applicants respectfully submit that the biological material is known to the public.

Furthermore, Applicants point out the highlighted section in Exhibit A showing that the cDNA sequence corresponding to the GM-CSF common beta chain sequences was deposited in the publicly accessible GenBank database (accession no. M38275). Therefore the sequence is readily available. Applicants respectfully submit that a person of ordinary skill in the art can make the publicly known D4β_c amino acid sequences without undue experimentation, and have them readily available in their possession.

Respectfully submitted,



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Biochemistry

Molecular cloning of a second subunit of the receptor for human granulocyte–macrophage colony-stimulating factor (GM-CSF): Reconstitution of a high-affinity GM-CSF receptor

(cytokine receptor/hemopoietic growth factor/hemopoiesis gene family)

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Communicated by George Palade, September 7, 1990

ABSTRACT **Using the mouse interleukin 3 (IL-3) receptor cDNA as a probe, we obtained a homologous cDNA (KH97) from a cDNA library of a human hemopoietic cell line, TF-1. The protein encoded by the KH97 cDNA has 56% amino acid sequence identity with the mouse IL-3 receptor and retains features common to the family of cytokine receptors. Fibroblasts transfected with the KH97 cDNA expressed a protein of 120 kDa but did not bind any human cytokines, including IL-3 and granulocyte–macrophage colony-stimulating factor (GM-CSF). Interestingly, cotransfection of cDNAs for KH97 and the low-affinity human GM-CSF receptor in fibroblasts resulted in formation of a high-affinity receptor for GM-CSF. The dissociation rate of GM-CSF from the reconstituted high-affinity receptor was slower than that from the low-affinity site, whereas the association rate was unchanged. Cross-linking of ^{125}I-labeled GM-CSF to fibroblasts cotransfected with both cDNAs revealed the same cross-linking patterns as in TF-1 cells—i.e., two major proteins of 80 and 120 kDa which correspond to the low-affinity GM-CSF receptor and the KH97 protein, respectively. These results indicate that the high-affinity GM-CSF receptor is composed of at least two components in a manner analogous to the IL-2 receptor. We therefore propose to designate the low-affinity GM-CSF receptor and the KH97 protein as the α and β subunits of the GM-CSF receptor, respectively.**

All hemopoietic cells ultimately arise from self-renewing pluripotent hemopoietic stem cells which are produced continuously in the bone marrow. Bone marrow stromal cells and a number of soluble factors, known as cytokines, play crucial roles in this process. Among these cytokines, interleukin 3 (IL-3), also known as multi-colony-stimulating factor (multi-CSF), stimulates early progenitor cells and supports the development of various cell lineages (1). While GM-CSF was initially defined as a factor that gives rise to granulocyte and macrophage colonies *in vitro*, recent evidence indicates that GM-CSF has broader biological activities, including stimulation of early hemopoietic progenitor cells and the development of other cell lineages (2, 3).

The biological effects of both GM-CSF and IL-3 are mediated by specific cell surface receptors. The human GM-CSF (hGM-CSF) receptor, cloned by Gearing *et al.* (4), exhibits low-affinity binding for GM-CSF when expressed on COS7 cells. Although there is evidence indicating that GM-CSF induces tyrosine phosphorylation (5), no tyrosine kinase consensus sequence was found (4). It is likely that the functional high-affinity GM-CSF receptor is composed of multiple subunits. Both GM-CSF and IL-3 induce tyrosine phosphorylation of a similar set of proteins (6–8) and they have overlapping biological activities (1–3). In addition, evidence indicates that the binding of hGM-CSF to its receptor is partially inhibited by human IL-3 (hIL-3) and vice versa (9–11). These results suggest that the hGM-CSF receptor and the hIL-3 receptor may share a common component.

Mouse IL-3 (mIL-3)-responsive cells express low- and high-affinity receptors for IL-3 (12–14). We recently isolated a cDNA (AIC2A) encoding a low-affinity mIL-3 binding protein which is a member of a recently identified cytokine receptor family (15). Although AIC2A does not contain a tyrosine kinase consensus sequence, AIC2A is a component of the high-affinity receptor (J. Schreurs and A.M., unpublished results). We also isolated a cDNA (AIC2B) which is highly identical (95% at the nucleotide level) to the IL-3 receptor cDNA (AIC2A) but is derived from a distinct gene (16). Despite its unusually high sequence identity with the IL-3 receptor, the AIC2B protein does not bind IL-3 and its function is currently unknown.

In this report, we present the cloning of a human cDNA which has homology with the mIL-3 receptor cDNA.§ The protein encoded by the cloned cDNA alone did not bind any of the cytokines tested. However, it conferred high-affinity binding for hGM-CSF when cotransfected with the low-affinity hGM-CSF receptor cDNA. This result indicates that the cloned IL-3 receptor-like cDNA (KH97) encodes a second subunit of the high-affinity hGM-CSF receptor.

MATERIALS AND METHODS

Construction of cDNA Library and Isolation of cDNA Clones. Poly(A)$^+$ RNA isolated from TF-1 cells (17) was converted to double-stranded cDNA by using oligo(dT) primers or specific primers corresponding to the cDNA sequence (Fig. 1). cDNA libraries were constructed either in the λgt11 phage vector or the simian virus 40-based mammalian expression vector pME18 (K. Maruyama and A.M., unpublished results). Using a ^{32}P-labeled mouse IL-3 receptor cDNA fragment (15) as a hybridization probe, we isolated a 3-kilobase (kb) human cDNA fragment (KH85) from the phage library under low-stringency conditions: hybridization at 42°C with 6× SSPE (1× SSPE is 150 mM NaCl/100 mM NaH_2PO_4/1 mM EDTA, pH 7.4) in the presence of 20% (vol/vol) formamide and washing at 50°C with 2× SSPE.

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Abbreviations: IL-2, IL-3, etc., interleukin 2, interleukin 3, etc.; GM-CSF, granulocyte–macrophage colony-stimulating factor; h-, human; m-, mouse.

‡To whom reprint requests should be addressed.

§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M38275).

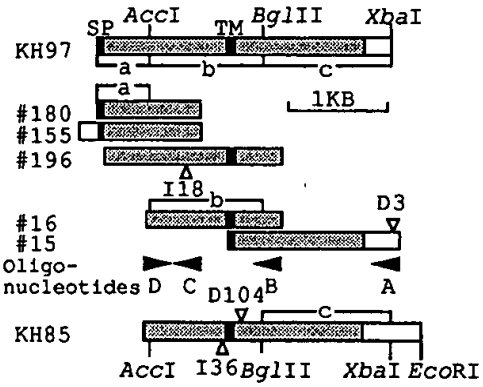

FIG. 1. The cloned cDNA fragments. Typical cDNA fragments obtained by using specific oligonucleotide primers are shown as bars, with the unshaded portion indicating the noncoding regions. The locations of oligonucleotides used to prime the cDNA synthesis are shown as arrowheads A, B, and C. An oligonucleotide corresponding to the region D and the KH85 cDNA fragment were used to isolate these cDNA fragments. All the cloned DNA fragments had the same sequence except for small insertions and deletions as indicated by I18 (an 18-base-pair insertion), I36 (a 36-base-pair insertion), D104 (a 104-base-pair deletion), and D3 (a 3-base-pair deletion). The KH97 cDNA was constructed with #180 (fragment a), #16 (fragment b), and KH85 (fragment c) as indicated. SP and TM indicate the signal peptide and the transmembrane domain, respectively. Scale indicates 1 kilobase.

The cDNA encoding the low-affinity hGM-CSF receptor was isolated by using the polymerase chain reaction primed with specific oligonucleotides corresponding to the 5′ untranslated and the 3′ untranslated regions of the published sequence (4). The cloned DNA fragment was sequenced to confirm the identity of the cDNA.

Transfection. Five micrograms of either individual plasmid DNA or a combination of two plasmid DNAs was transfected into semiconfluent COS-7 cells (African green monkey kidney cells expressing the T antigen of simian virus 40) by the DEAE-dextran method as described previously (18). Three days after transfection, COS-7 cells were harvested and analyzed by ligand binding assays or chemical cross-linking experiments. NIH 3T3 mouse cells were stably transfected, using the *neo* gene as a selection marker, by the calcium phosphate procedure (19). Stable transfectants were selected with G418 at 1 mg/ml.

Radioiodination of hGM-CSF and Binding Experiments. *Escherichia coli*-derived hGM-CSF iodinated with Bolton–Hunter reagent (^{125}I-GM-CSF) was used for binding assays as described previously (20). Dissociation constants were obtained by the LIGAND program (21). Chemical cross-linking was performed with 0.2 mM disuccinimidyl suberate on transfected COS-7 cells (10^6 cells) or NIH 3T3 stable transfectants (3×10^6 cells) preincubated with 4 nM ^{125}I-GM-CSF. Proteins were analyzed as described previously (20).

RESULTS

Isolation and Characterization of a Human cDNA Homologous to the mIL-3 Receptor cDNA. Using the mIL-3 receptor cDNA as a probe, we screened a cDNA library made from a human erythroleukemic cell line, TF-1, which responds to multiple human factors, including IL-3, IL-4, IL-5, GM-CSF, and erythropoietin (17). A cDNA clone (KH85) homologous to the mIL-3 receptor cDNA (approximately 70% identical at the nucleotide level) was obtained from about 4×10^5 independent clones. This clone lacks about 600 bases from its 5′ end compared with the sequence of the mouse cDNA. We therefore prepared cDNA libraries by using specific primers based on the KH85 sequence and screened these libraries with the KH85 probes (Fig. 1). We found only one type of cDNA among 26 positive clones analyzed. Although several cDNAs with an insertion and/or a deletion were isolated (Fig. 1), these cDNAs seemed to be created by alternative splicing rather than encoded by a distinct gene, because the insertions and deletions were found at sites corresponding to the exon–intron junctions of the mouse AIC2 genes (D.M.G., unpublished results). We reconstructed a cDNA (KH97) encoding the entire protein (Fig. 1) and used this for further studies.

Comparison of the amino acid sequence encoded by the KH97 cDNA with that of the AIC2A and AIC2B proteins showed 56% and 55% identity, respectively (Fig. 2). The homology was distributed throughout the coding region in

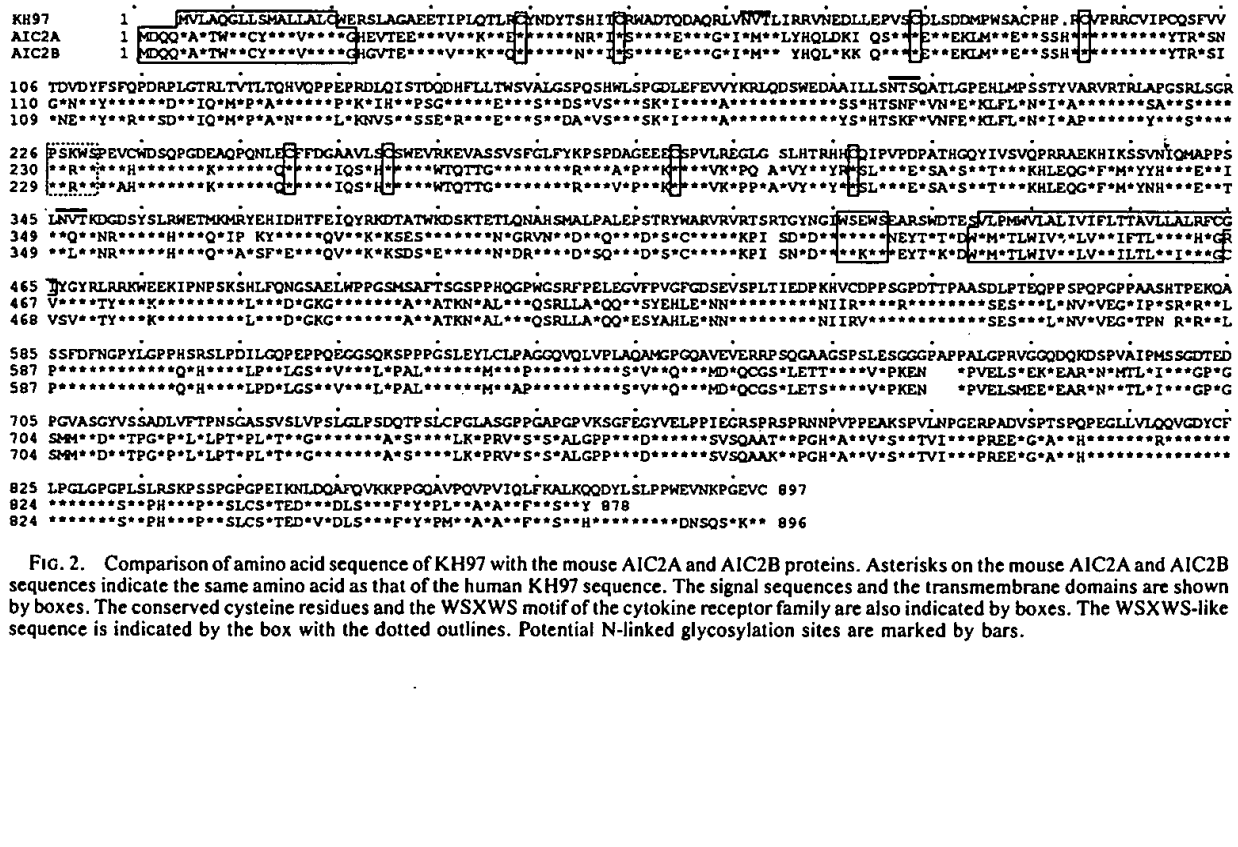

FIG. 2. Comparison of amino acid sequence of KH97 with the mouse AIC2A and AIC2B proteins. Asterisks on the mouse AIC2A and AIC2B sequences indicate the same amino acid as that of the human KH97 sequence. The signal sequences and the transmembrane domains are shown by boxes. The conserved cysteine residues and the WSXWS motif of the cytokine receptor family are also indicated by boxes. The WSXWS-like sequence is indicated by the box with the dotted outlines. Potential N-linked glycosylation sites are marked by bars.

both cases. The AIC2B protein has an extra 18 amino acids at the C terminus compared with the mIL-3 receptor (AIC2A), and the KH97 protein also has the extra 18 amino acids (Fig. 2). Because of the high sequence similarity between AIC2A and AIC2B (16), it is not clear to which of the mouse genes the KH97 cDNA corresponds.

The KH97 mRNA was detected in the myelogenous leukemic cell lines TF-1 and KG1 but not in the NK cell line YT or the mouse IL-3- and GM-CSF-dependent cell line PT18 under stringent hybridization conditions (Fig. 3).

Expression of KH97 in COS-7. A transient expression system using COS-7 cells was used to evaluate expression of the KH97 cDNA. Because of its extensive sequence similarity to the mIL-3 receptor cDNA, we examined whether hIL-3 could bind to COS-7 cells transfected with the KH97 cDNA. Using up to 20 nM ^{125}I-hIL-3, we could not detect any specific binding. We also constructed full-length cDNAs of KH97 variants (Fig. 1), which were presumably derived from alternative splicing, and tested the binding of hIL-3 to transfected COS-7 cells. However, we did not find any specific binding. We then examined the binding of other cytokines. However, hIL-2 (1 nM), hIL-4 (1 nM), hIL-5 (5 nM), hGM-CSF (20 nM), and human erythropoietin (10 nM) all showed no specific binding to KH97-transfected COS-7 cells at the indicated concentrations. To exclude the possibility that COS-7 cells failed to express the protein encoded by the KH97 cDNA, we prepared antibodies against a peptide encoded by the KH97 cDNA and used them to detect the KH97 cDNA-encoded protein in COS-7 cells. Western blotting using anti-peptide antibodies confirmed the expression of a 120-kDa protein in the KH97 cDNA-transfected COS-7 cells (data not shown). We therefore concluded that the KH97 protein did not bind any cytokines examined when expressed in COS-7 cells.

Cotransfection of the KH97 cDNA and the GM-CSF Receptor cDNA. Although the KH97 protein did not bind any of the cytokines we examined, there still remained the possibility that it is a component of another known or unknown cytokine receptor. It has been shown that the β chain of the IL-2 receptor does not bind IL-2 when expressed in COS-7 cells, but it does bind IL-2 with intermediate affinity in Jurkat cells (22). Furthermore, coexpression of the low-affinity IL-2 receptor (α chain) and the β chain forms a high-affinity binding site (22). These observations indicate that the binding of a ligand to its receptor can be determined by the interaction of multiple proteins. Combining this idea with the observation that the IL-3 and GM-CSF receptors may share a common element (9–11), we considered the possibility that the KH97 protein is a subunit of the hGM-CSF receptor. To examine this possibility, we cotransfected the KH97 cDNA with the low-affinity GM-CSF receptor cDNA (4).

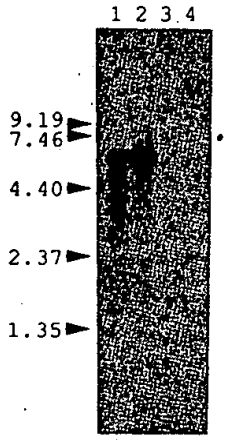

FIG. 3. Northern blotting of poly(A)$^+$ RNA from various cell lines. Three micrograms of each poly(A)$^+$ RNA was electrophoresed on a 1% agarose gel, transferred to a nitrocellulose membrane, and hybridized with the ^{32}P-labeled KH97 cDNA. Lane 1, TF-1; lane 2, KG-1; lane 3, YT; and lane 4, PT18. Sizes are given in kb.

We examined the equilibrium binding of hGM-CSF to COS-7 cells transfected with the hGM-CSF receptor cDNA, the KH97 cDNA, or a combination of these cDNAs (Fig. 4). The hGM-CSF receptor expressed exclusively low-affinity binding sites (K_d = 3.2 nM) as reported by Gearing *et al.* (4), whereas the KH97 protein alone did not express any detectable binding for GM-CSF. However, cotransfection of these two cDNAs resulted in the expression of both high- (120 pM) and low- (6.6 nM) affinity binding sites.

The same results were obtained with stable transfectants of NIH 3T3 cells (Fig. 4): hGM-CSF receptor cDNA-transfected NIH 3T3 cells bound hGM-CSF with K_d = 2.7 nM, whereas the NIH 3T3 transfected with both the hGM-CSF receptor and the KH97 cDNAs bound hGM-CSF with K_d = 170 pM. Because of the low expression of the low-affinity hGM-CSF receptor compared with the KH97 protein in this NIH 3T3 transfectant, no statistically significant low-affinity binding site was found by using the LIGAND program (21). Again no specific binding of hGM-CSF was detected in the cells transfected with the KH97 cDNA alone.

In both COS-7 and NIH 3T3 cells, binding of ^{125}I-hGM-CSF was blocked by hGM-CSF but not by hIL-3. In addition, cotransfection of the KH97 cDNA with the human IL-2 receptor α chain cDNA or the human IL-4 receptor cDNA did not change the binding affinity of their respective ligands. Cotransfection of the cDNAs encoding the low-affinity hGM-CSF receptor with that for the IL-2 receptor β chain, the IL-4 receptor, mouse AIC2A, or AIC2B also did not change the affinity for hGM-CSF. Thus, formation of the high-affinity GM-CSF receptor is specific to the combination of the low-affinity GM-CSF receptor and the KH97 protein.

We then analyzed the binding kinetics to determine whether the high-affinity binding of hGM-CSF to cotransfected cells was due to an increased rate of association or a decreased rate of dissociation. As shown in Fig. 5, GM-CSF

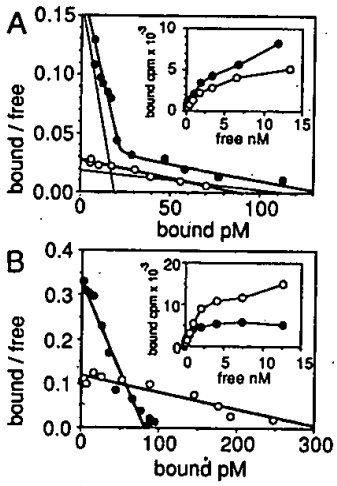

FIG. 4. Binding of ^{125}I-hGM-CSF. (*A*) Duplicate suspensions of 2 × 10^5 COS-7 cells transiently transfected with the hGM-CSF receptor and KH97 cDNAs were used for ^{125}I-hGM-CSF binding assays. (*B*) Duplicate suspensions of NIH 3T3 stable transfectants (5 × 10^6 cells) were used for binding assays. Binding assays were performed at 4°C. ○, Cells transfected with the hGM-CSF receptor cDNA and the vector DNA. ●, Cells cotransfected with the hGM-CSF receptor cDNA and the KH97 cDNA. Scatchard plots of the binding data are shown. (*Insets*) Equilibrium binding profiles.

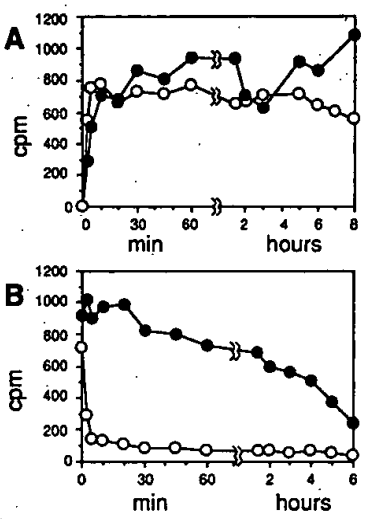

FIG. 5. Kinetics of binding of ^{125}I-hGM-CSF to the receptor. (*A*) Association rate. NIH 3T3 stable transfectants were incubated with 200 pM ^{125}I-hGM-CSF at 4°C for various times and the cell-bound radioactivity was measured. (*B*) Dissociation rate. A 200-fold excess of unlabeled hGM-CSF was added to the NIH 3T3 stable transfectants, which were preincubated with 200 pM ^{125}I-hGM-CSF for 4 hr at 4°C, and the residual cell-bound radioactivity was then measured at various times at 4°C. ○, NIH 3T3 cells expressing the low-affinity hGM-CSF receptor. ●, NIH 3T3 cells expressing both the hGM-CSF receptor and the KH97 protein.

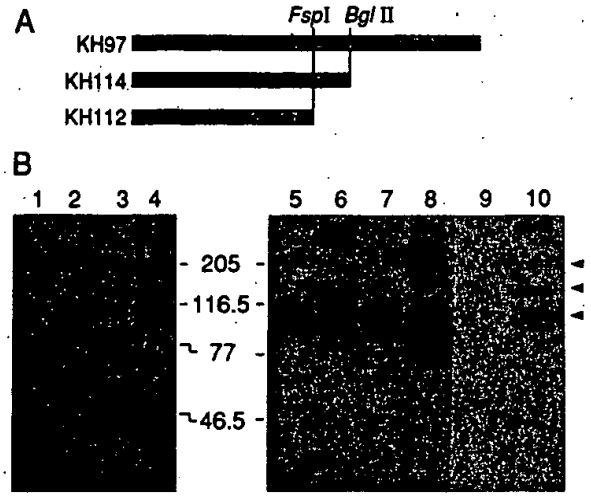

FIG. 6. Expression of GM-CSF receptors on COS-7 cells and NIH 3T3 cells. (*A*) Structure of the cDNAs encoding the KH97 protein and its deletion mutants. KH114 is truncated at the *Bgl* II site and KH112 is truncated at the *Fsp* I site (Fig. 1). (*B*) Cross-linking of ^{125}I-hGM-CSF to the transfected COS-7 cells or NIH 3T3 stable transformants. Lanes 1–4, NIH 3T3 cells; lanes 5–9, COS-7 cells. Cells were transfected with the following: lane 1, mock transfection; lane 2, GM-CSF receptor cDNA; lane 3, KH97 cDNA; lane 4, GM-CSF receptor cDNA and KH97 cDNA; lane 5, GM-CSF receptor cDNA; lane 6, GM-CSF receptor cDNA and KH97 cDNA; lane 7, GM-CSF receptor cDNA and KH114 cDNA; lane 8, GM-CSF receptor cDNA and KH112 cDNA; and lane 9, KH97 cDNA. Lane 10, cross-linking with TF-1 cells. Specific bands detected in COS-7 cells transfected with deletion mutants are marked by dots (lanes 6–8). Protein masses are given in kDa.

binding increased with similar kinetics in both hGM-CSF receptor cDNA-transfected cells and cotransfected cells. In dissociation experiments, the addition of unlabeled hGM-CSF to cells preequilibrated with ^{125}I-hGM-CSF led to the rapid release of radioligand from the hGM-CSF receptor-transfectants ($t_{1/2}$ = 2 min), and a slow release ($t_{1/2}$ = 290 min) from the cells expressing both proteins. These results indicate that the high-affinity binding of hGM-CSF to the cotransfected cells is due to the slow dissociation of hGM-CSF from the receptor.

Cross-Linking of hGM-CSF to the KH97 Protein. To test if the KH97 protein actually binds hGM-CSF when coexpressed with the low-affinity hGM-CSF receptor in COS-7 or NIH 3T3 cells, we performed chemical cross-linking experiments using ^{125}I-hGM-CSF (Fig. 6). Cross-linking of ^{125}I-hGM-CSF to the low-affinity hGM-CSF receptor-expressing cells showed only one band of 95 kDa (Fig. 6*B*, lane 5), whereas no specific band was detected in cells expressing only the KH97 protein (Fig. 6*B*, lane 9). However, cross-linking of the cells expressing both proteins revealed three bands, of 95, 135, and 210 kDa (Fig. 6*B*, lane 6). This cross-linking pattern was identical to that obtained with TF-1 cells (Fig. 6*B*, lane 10). These bands were not detected when cross-linking was performed in the presence of an excess of unlabeled hGM-CSF (data not shown). Subtraction of the molecular mass of hGM-CSF from the molecular mass of the cross-linked proteins results in calculated masses of 80, 120, and 195 kDa, which correspond, respectively, to the low-affinity hGM-CSF receptor, the KH97 protein, and possibly a complex of the two. Similar results were obtained with NIH 3T3 transfectants (Fig. 6*B*, lanes 1–4).

Cross-linking of hGM-CSF to the KH97 protein was further confirmed by making cytoplasmic domain deletion mutants of KH97 (Fig. 6*A*). Deletion mutants of the KH97 cDNA were cotransfected with the low-affinity hGM-CSF receptor cDNA into COS-7 cells. Whereas the band at 95 kDa was not changed, the band at 135 kDa was shifted to lower molecular masses in cells transfected with these deletion mutants (Fig. 6, lanes 6–8, marked by dots). The shift of the molecular mass was consistent with the shift of the bands revealed by Western blotting using anti-peptide antibodies against the KH97 protein (data not shown). These results clearly indicate that the KH97 protein is cross-linked with hGM-CSF when coexpressed with the low-affinity hGM-CSF receptor. In addition, this experiment demonstrates that formation of the high-affinity receptor for hGM-CSF does not require the cytoplasmic domain of the KH97 protein.

DISCUSSION

Our results demonstrate that the low-affinity hGM-CSF receptor together with the KH97 protein forms a high-affinity receptor for hGM-CSF. This is analogous to the formation of a high-affinity receptor for IL-2 by coexpression of the α and β chains of the IL-2 receptor. The α chain of the IL-2 receptor binds IL-2 with low affinity (23, 24), whereas the β chain by itself does not bind IL-2 when expressed in COS-7 cells (22). However, coexpression of both chains leads to the formation of a high-affinity receptor, and both chains can be cross-linked with IL-2 (22). Because of similarity between the IL-2 and the hGM-CSF high-affinity receptors, we propose to designate the low-affinity hGM-CSF receptor as the α chain and the KH97 protein as the β chain of the hGM-CSF receptor.

It is of particular interest that the KH97 cDNA was isolated on the basis of its homology to the mIL-3 receptor. Among various cytokines the sequence conservation of IL-3 between mouse and human is unusually weak (only 29% identity at the amino acid level) (25). Nevertheless we were able to isolate a human cDNA which was homologous (56% identity at the amino acid level) to the mIL-3 receptor cDNA. However, as described above, we were unable to demonstrate IL-3 bind-

ing to the KH97 protein expressed on fibroblasts. Recently, we isolated a second mouse gene (AIC2B) which is highly homologous to the mIL-3 receptor gene (AIC2A), but the AIC2B protein does not bind mIL-3 (16). One possibility is that the KH97 protein is the human protein corresponding to the mouse AIC2B protein and therefore cannot bind IL-3. If this is the case, there may exist an AIC2A-like human protein which binds hIL-3. To address this question we have extensively searched for additional cDNA clones which might hybridize with either the mouse AIC2A or the human KH97 cDNA probe. However, we could not find any cDNA that is homologous to, yet distinct from, the KH97 cDNA. Since the abundance of the AIC2B mRNA is generally higher than that of the AIC2A (mIL-3 receptor) mRNA in mouse (16), the failure to identify the hIL-3 receptor cDNA may be due to its low abundance.

Another possibility is that, unlike mice, humans do not have two homologous genes. In this case, the IL-3 binding protein, like IL-3, may have only weak conservation between human and mouse. Alternatively, the KH97 protein may be a component of both the hIL-3 and hGM-CSF receptors. IL-3 and GM-CSF induce tyrosine phosphorylation of similar sets of proteins (5) and they have overlapping biological activities (1–3). In addition, binding of hGM-CSF to its receptor is partially blocked by hIL-3 and vice versa (9–11), although IL-3 and GM-CSF have no structural homology. The KH97 protein may be shared between the hIL-3 receptor and the hGM-CSF receptor—i.e., the KH97 protein forms the high-affinity receptor for hGM-CSF with the α chain of the hGM-CSF receptor and it also forms the high-affinity receptor for hIL-3 with an unidentified protein which may or may not bind hIL-3 by itself. If this is the case, the cross-competition as well as overlapping biological activities of the two factors may be explained. It is of interest that no cross-competition of binding between mIL-3 and mGM-CSF has yet been reported. If the AIC2A protein forms the high-affinity mIL-3 receptor with an unidentified protein and the AIC2B protein is the β chain of the mGM-CSF receptor, then there may be no cross-competition between mouse factors. As the mouse low-affinity receptor for GM-CSF has not yet been isolated, at present we are not able to test this hypothesis by using cotransfection with either the AIC2A or the AIC2B cDNA. We have examined the possibility that the low-affinity hGM-CSF receptor may form a high-affinity receptor with either the mouse AIC2A or the AIC2B protein; however, none of these combinations resulted in high-affinity binding with either mouse or human GM-CSF. If there is another human AIC2 homologue which binds hIL-3 in a manner analogous to mouse AIC2A, cross-competition between hIL-3 and hGM-CSF may occur due to another shared component. If this component is present abundantly in mouse, cross-competition may not be observed. In any case, it is important to find whether humans have two AIC2 homologues and also whether mouse AIC2B is the β chain of the mGM-CSF receptor.

Neither the α nor the β chain of the GM-CSF receptor has a tyrosine kinase consensus sequence, and GM-CSF did not induce tyrosine phosphorylation in the NIH 3T3 transfectants expressing the α and β subunits of the GM-CSF receptor (T.K., unpublished data), yet GM-CSF induces tyrosine phosphorylation in hemopoietic cells (5). Thus, signal transduction through the GM-CSF receptor must require additional component(s). To understand the molecular mechanisms of signal transduction it is of particular importance to identify those additional component(s) required for signal transduction.

During the preparation of this manuscript, Metcalf *et al.* (26) reported that transfection of the α subunit of hGM-CSF receptor cDNA in a mGM-CSF-dependent mouse cell line resulted in only low-affinity binding for hGM-CSF and a high concentration of hGM-CSF stimulated proliferation. Inability of the human α subunit to form a high-affinity receptor in mouse cells is consistent with our result that cotransfection of the human α subunit cDNA and the mouse AIC2A or AIC2B cDNA did not result in a high-affinity binding for hGM-CSF in COS-7 cells. However, the mechanism by which the low-affinity hGM-CSF receptor transmits a growth signal remains unclear.

We are grateful to J. Schreurs, G. Hsu, and P. Hung for preparing the affinity-purified anti-peptide antibodies and to F. Vega and D. Robison for oligonucleotide synthesis. We thank R. Kastelein and A. Shanafelt for their help in cloning the low-affinity GM-CSF receptor cDNA and H.-M. Wang and J. Schreurs for helpful discussion. DNAX Research Institute of Molecular and Cellular Biology is supported by Schering-Plough Corporation.

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Histidine-367 of the human common β chain of the receptor is critical for high-affinity binding f human granul cyte–macrophage colony-stimulating factor

(growth factor/receptor/ligand binding/functional analysis)

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ABSTRACT **High-affinity receptors for granulocyte–macrophage colony-stimulating factor (GM-CSF), interleukin 3, and interleukin 5 consist of ligand-specific α chains (low-affinity subunits) and a common β chain (β_c) that converts each complex to a high-affinity form. Although β_c alone has no detectable cytokine-binding activity, amino acid substitutions for Glu-21 of human GM-CSF significantly reduce high-affinity but not low-affinity binding, implying that β_c interacts directly with GM-CSF during formation of the high-affinity receptor but only in the presence of the α chain. A potential GM-CSF-binding determinant was identified in the second hemopoietin domain of β_c, and the role of individual residues within this region was investigated by determining the ability of mutated β_c chains to confer high-affinity binding when coexpressed with the α subunit of the GM-CSF receptor in COS cells. Substitutions involving Met-363, Arg-364, Tyr-365, and Glu-366 did not affect high-affinity binding. However, substitution of His-367 by lysine or glutamine abolished high-affinity binding, suggesting that this residue may form an important part of the high-affinity GM-CSF-binding determinant. Consistent with the loss of high-affinity binding, higher concentrations of human GM-CSF were required to stimulate proliferation of CTLL-2 cell lines transfected with cDNAs for GM-CSF receptor α chain and His-367 β_c mutant than those expressing GM-CSF receptor α subunit and β_c wild type.**

Mammalian granulocyte–macrophage colony-stimulating factor (GM-CSF) promotes the survival, proliferation, maturation, and functional activation of a spectrum of hemopoietic lineages, including committed myeloid progenitors, neutrophils, monocytes, and eosinophils (1). Many of the biological activities of GM-CSF are shared by interleukin 3 (IL-3) and a subset of these activities, such as the ability to stimulate eosinophil production, are also common to interleukin 5 (IL-5) (2).

The biological responses induced by GM-CSF, IL-3, and IL-5 are initiated after engagement of the cytokines with specific cell-surface receptors expressed on the target cells. The human receptors are heterodimers composed of the cytokine-specific α subunits, GM-CSF receptor α subunit (GM-CSFRα), IL-3 receptor α subunit (IL-3Rα), or IL-5 receptor α subunit (IL-5Rα), which bind ligand with low affinity, and a common β chain (β_c), which has no detectable binding activity alone but converts the low-affinity receptor–ligand complexes to high-affinity forms (3–6). Cross-linking studies suggest that β_c contacts GM-CSF, or is very close to it, in the high-affinity receptor complex. Murine AIC2B protein (7) is functionally equivalent to human β_c, as it lacks cytokine-binding activity alone and confers high-affinity binding when coexpressed with murine GM-CSFRα, murine IL-3Rα, and murine IL-5Rα (4, 8–10). Conversely, murine AIC2A protein [or IL-3-specific β chain (β_{IL-3})], which has no known functional equivalent in humans, confers high-affinity binding only to murine IL-3Rα but differs from AIC2B protein and β_c in that it possesses intrinsic IL-3-binding activity (9, 11).

Transfection studies with the factor-dependent myeloid and lymphoid murine cell lines FDC-P1 and CTLL-2, respectively, indicate that both the α and β subunits of the receptors for human GM-CSF (hGM-CSF), IL-3, and IL-5 are essential for biological responsiveness to the corresponding cytokines (4, 10, 12, 13). However, signaling can occur at high nonphysiological concentrations of hGM-CSF or human IL-3 in the absence of high-affinity binding if the cells express human GM-CSFRα and murine AIC2B (4, 12, 13).

The GM-CSFRα, IL-3Rα, IL-5Rα, and β_c subunits are members of the cytokine-receptor superfamily and contain either a single extracellular hemopoietin domain (the α subunits) or dual hemopoietin domains (β_c) (14, 15). The structure of hemopoietin-domain receptors is epitomized by the extracellular binding domain of the human growth hormone (hGH) receptor (hGHR), which was determined by x-ray crystallographic analysis of the hGH–hGHR complex (16). This complex consists of two identical receptor subunits and a single molecule of hGH and is formed by sequential interaction of the first hGHR subunit with site 1 on hGH, followed by binding of the second hGHR subunit to site 2 on hGH and an additional site on the first hGHR subunit (17). This model of sequential receptor dimerization is thought to apply to other members of the cytokine receptor family. Thus, human and murine GM-CSFRα, IL-3Rα, and IL-5Rα are considered analogous to the hGHR subunit that binds site 1 of hGH, whereas human β_c and murine AIC2A and AIC2B subunits are functionally equivalent to the hGHR subunit that binds site 2 of hGH.

Despite the inability of β_c to bind GM-CSF, IL-3, or IL-5 independently of the corresponding α subunits, amino acid substitutions of Glu-21 of hGM-CSF impair high- but not low-affinity binding, suggesting that, within the complex, Glu-21 may interact directly with β_c (18, 19). To investigate which residues of human β_c are required for high-affinity binding of GM-CSF, a series of β_c mutants with various amino acid substitutions in the extracellular domain were generated by site-directed mutagenesis and examined for

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Abbreviations: GM-CSF, granulocyte–macrophage colony-stimulating factor; hGM-CSF, human GM-CSF; GM-CSFRα, α chain of GM-CSF receptor; IL-2, IL-3, and IL-5, interleukin 2, 3, and 5, respectively; β_c, common β subunit of GM-CSF, IL-3, and IL-5 receptors; hGH, human growth hormone; hGHR, hGH receptor; IL-3Rα and IL-5Rα, α subunits of IL-3 and IL-5 receptors, respectively.

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their capacity to contribute to high-affinity binding when coexpressed with GM-CSFRα in COS cells. The functional activity of β_c wild-type and β_c H367K mutant, which have normal and impaired high-affinity binding activity, respectively, was assessed by stable coexpression of these forms of β_c with GM-CSFRα in CTLL-2 cells.

MATERIALS AND METHODS

Isolation of Human β_c cDNA. A cDNA encoding β_c was assembled from three separate fragments obtained from overlapping λgt11 cDNA clones. Each λ clone was isolated from a human placental cDNA library (Clontech) by hybridization at high stringency (20) with two ^{32}P-labeled DNA fragments corresponding to the first and last 0.4 kb of the β_c coding region that were initially amplified directly from the placental cDNA library using the PCR. The nucleotide sequence of the composite cDNA was determined by using a T7 DNA polymerase sequencing kit (Promega) and found to contain an additional 42 bp of 5′ untranslated region compared with the β_c (formerly clone KH97) cDNA sequence as reported (6).

Expression Constructs and Site-Directed Mutagenesis. Human GM-CSFRα (3) and β_c cDNAs were subcloned into pCDM8 (21) (Invitrogen) to generate the COS cell expression constructs pCDM8-α and pCDM8-β_c, respectively. A series of pCDM8-β_c constructs encoding mutant forms of β_c with the amino acid substitutions shown in Fig. 1*B* were generated by using the PCR overlap/splice technique (22) with *Pfu* DNA polymerase (Stratagene) and pCDM8-β_c as the DNA template. The mutated PCR products were digested with *Bsa* I and *Bss*HII and substituted for the corresponding wild-type *Bsa* I–*Bss*HII fragment of pCDM8-β_c, and the mutations were validated by nucleotide sequencing. For expression in CTLL-2 cells, cDNAs for GM-CSFRα and wild-type β_c or mutant subunits were inserted into the pEF-BOS expression vector (25) to generate pEF-BOS-α and pEF-BOS-β_c (wild-type and mutant derivatives).

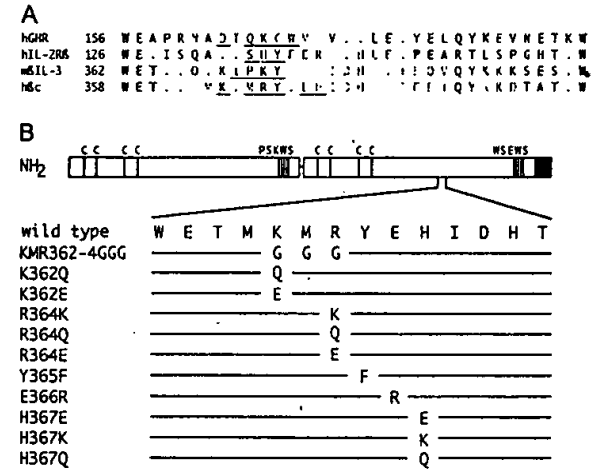

FIG. 1. Identification and mutagenesis of residues in β_c predicted to interact with hGM-CSF. (*A*) Sequence alignment of residues in the predicted loop between the C-terminal B and C strands within the proposed double β-barrel structural models of hGHR (16), human IL-2R β chain (hIL-2Rβ) (23), mouse IL-3R β subunit (mβIL-3) (24), and hβ_c. Residues shown to participate in ligand binding by hGHR, hIL-2Rβ, and mβ_{IL-3} and predicted to contribute to ligand binding by β_c are underlined. Amino acids are numbered at left, identical residues are shaded, and gaps introduced to enhance the alignment are indicated by dots. (*B*) Schematic representation of the dual hemopoietin domains (rectangles) and transmembrane domain (filled box) of hβ_c, indicating the approximate position of conserved cysteines and WSXWS-like motifs. Sequences corresponding to the predicted loop between the B and C strands of wild-type and mutant forms of hβ_c are indicated.

Cells and Transfections. COS cells (26) were grown in RPMI 1640 medium/10% bovine serum, and CTLL-2 cells (27) were grown in RPMI 1640/10% fetal calf serum/50 μM 2-mercaptoethanol/2 mM glutamine/glucose at 1 mg/ml/human interleukin 2 (IL-2) at 100 international units/ml (Cetus).

For transient expression analysis, 1–2 × 10^7 COS cells suspended in 0.8 ml of phosphate-buffered saline (PBS) were mixed with 5 μg of pCDM8-α alone or 5 μg of pCDM8-α and 20 μg of pCDM8-β_c (wild type or mutant) and transfected by electroporation at 0.3 kV and 500 μF in a 0.4-cm cuvette (Bio-Rad).

Stably transfected CTLL-2 cell lines were generated in two stages. Approximately 3 × 10^7 CTLL-2 cells were cotransfected with 20 μg of pEF-BOS-α and 2 μg of PGK (for phosphoglycerate kinase promoter)-hygro plasmids (from M. Hibbs, Ludwig Institute, Melbourne, Australia) by electroporation at 0.2 kV and 960 μF and selected in medium containing hygromycin sulfate at 200 μg/ml (Boehringer Mannheim). After 10–14 days, hygromycin-resistant cells were cloned by limiting dilution. One cell line, CTLLα1, expressed low-affinity receptors for hGM-CSF on the basis of flow cytometric analysis with the 2B7-17A monoclonal antibody against human GM-CSFRα (28). CTLLα1 cells were cotransfected with 20 μg of pEF-BOS-β_c (encoding wild-type β_c or mutant H367K) and 2 μg of PGK-neo (from B. Mann, Ludwig Institute) and selected in G418 (400 μg/ml) to generate the cell lines CTLL$\alpha\beta$.3 and CTLL$\alpha\beta$.4 (expressing wild-type β_c) and lines CTLL$\alpha\beta$10.1 and CTLL$\alpha\beta$10.2 (expressing β_c mutant H367K).

Equilibrium Binding Analysis. COS cells were harvested 72 hr after transfection by treatments with 0.04 M EDTA in HRF binding medium (10 mM Hepes-buffered RPMI 1640 medium/10% bovine serum) and chondroitin sulfate at 200 μg/ml (Sigma) in HRF medium. hGM-CSF was labeled to a specific radioactivity of 4.2 × 10^5 cpm/pmol by the iodine monochloride method (29). Duplicate samples of 2.5 × 10^5 cells in HRF medium were incubated with increased doses of ^{125}I-labeled hGM-CSF in the presence or absence of 830 nM hGM-CSF for 3 hr at 4°C. Specific binding was determined as described (3, 29), and Scatchard transformations of the saturation-binding data were generated by using EBDA and LIGAND computer software (Biosoft, Cambridge, U.K.) (30).

Indirect Immunofluorescence Analysis. Approximately 10^6 transfected COS cells were incubated in the presence of 20 μg of CRS-1, a rat monoclonal antibody against β_c (31) (from A. Miyajima, DNAX) at 4°C for 45 min. Cells were washed twice and incubated with 1 μg of fluorescein isothiocyanate-conjugated anti-rat immunoglobulin (Silenus, Melbourne, Australia) at 4°C for 30 min. Cell-surface expression of β_c was detected by fluorescence microscopy.

Proliferative Assays. Stably transfected CTLL-2 cell lines were washed three times in growth medium lacking exogenous human IL-2, and duplicate samples of 200 cells were inoculated into 60-well microtiter plates, in the presence or absence of increased concentrations of hGM-CSF (or human IL-2). Viable cells were counted 48 hr later. Maximal stimulation was defined as an average of ≥200 cells per well.

RESULTS

Mutagenesis of β_c cDNA. The second hemopoietin domain of β_c was considered likely to contain the hGM-CSF-binding site on the basis of mutational analysis of murine AIC2A protein (mβ_{IL-3}), which showed that the corresponding domain of this structurally related and functionally similar protein is essential for murine IL-3 binding (24). Possible

binding determinants of β_c were further localized by sequence alignment of the second hemopoietin domain of β_c with the single hemopoietin domains of the hGHR, the human IL-2 receptor β subunit, and the second hemopoietin domain of murine AIC2A ($m\beta_{IL\text{-}3}$); three members of the cytokine receptor family for which ligand-binding determinants have been identified by x-ray crystallography (16) or mutational analysis (23, 24). A sequence alignment of the pertinent region is shown in Fig. 1*A*, and the residues in this region critical for binding hGH, human IL-2, and murine IL-3 and those predicted to engage hGM-CSF are underlined. In the hGHR, this region forms a loop joining the B and C strands of the C-terminal β-barrel structure (16). It is this region of the hGHR that participates in binding site 2 of hGH after the first hGHR subunit has interacted with site 1 of hGH, and it is, therefore, formally equivalent to the region of β_c that interacts with hGM-CSF after formation of the low-affinity hGM-CSF–GM-CSFRα complex. Moreover, this loop in hGHR contacts a residue (Arg-19) in the N-terminal helix of hGH that is in a similar position to Glu-21 of hGM-CSF.

Site-directed mutagenesis was used to generate several cDNAs encoding mutant forms of β_c in which individual residues or groups of residues within this cluster were replaced by others with similar or distinct biochemical properties (Fig. 1*B*). Three basic residues, Lys-362, Arg-364, and His-367, were considered particularly strong candidates, as they could potentially form salt bridges with Glu-21 of hGM-CSF, a residue shown (18, 19) to be critical for high-affinity binding to the hGM-CSF receptor.

Binding Properties of β_c Mutants. To determine whether the substitutions within the second hemopoietin domain of β_c affected high-affinity binding, COS cells were cotransfected with expression vectors encoding human GM-CSFRα (3) and wild-type or mutant forms of β_c; the ability of the transfectants to bind ^{125}I-labeled hGM-CSF with high- and low-affinity was then determined. Scatchard plots corresponding to equilibrium-binding analysis of eight representative COS cell transfectants are shown in Fig. 2. Table 1 summarizes the raw data obtained from two separate experiments in which the wild-type and all β_c mutants were tested.

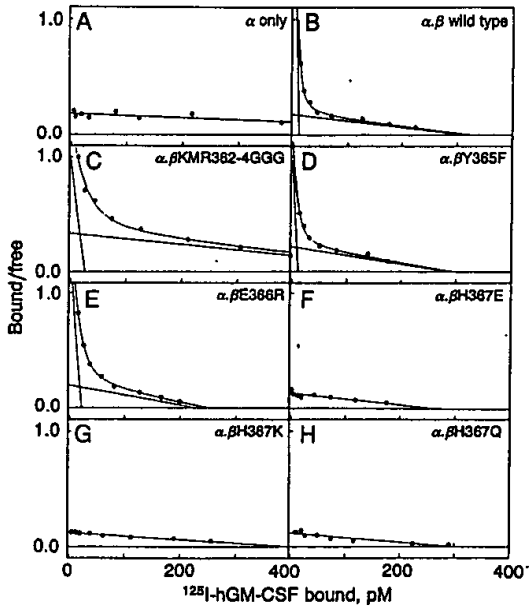

FIG. 2. Scatchard plots of ^{125}I-labeled hGM-CSF saturation-binding analysis of COS cell transfectants. Binding assays were performed 72 hr after the cells were transfected with cDNAs for GM-CSFRα (*A*), GM-CSFRα and β_c wild-type (*B*) or GM-CSFRα and the β_c mutants KMR362-4GGG (*C*), Y365F (*D*), E366R (*E*), H367E (*F*), H367K (*G*), or H367Q (*H*).

As expected, only low-affinity hGM-CSF-binding sites were detected on cells transfected with human GM-CSFRα cDNA (Fig. 2*A*), whereas both high- and low-affinity binding sites were detected on cells coexpressing GM-CSFRα and wild-type β_c (Fig. 2*B*) (3, 6). High- and low-affinity sites were also present on cells coexpressing GM-CSFRα and the β_c mutants, KMR362-4GGG (in which Lys-362–Met-363–Arg-364 was changed to Gly–Gly–Gly), Y365F (Tyr-365 → Phe), or E366R (Glu-366 → Arg) (Fig. 2 *C–E*). These results strongly suggest that the residues Lys-362, Met-363, Arg-364, and Tyr-366 are not directly involved in high-affinity binding and probably do not serve an important structural role.

In one experiment, low- and high-affinity sites were detected on cells cotransfected with cDNAs for GM-CSFRα and the β_c mutant H367E (containing a His-367 → Glu substitution), whereas in a second experiment only low-affinity sites could be detected (Table 1 and Fig. 2*F*). The degree of certainty associated with the prediction of both high- and low-affinity binding sites, rather than low-affinity binding sites alone, was determined when fitting curves to the equilibrium binding data, and it is notable that in the experiment where two sites were predicted, the *P* value was only 0.04 (Table 1). By contrast, in both experiments, no high-affinity receptors were detected on cells transfected with the β_c mutants H367K or H367Q (His-367 → Lys or His-367 → Gln, respectively) (Fig. 2 *G–H*), suggesting that these substitutions ablate high-affinity binding, either by destroying a

Table 1. Equilibrium dissociation constants, receptor numbers, and probability estimates for high- and low-affinity receptors for hGM-CSF on COS cells

	Low-affinity receptors		High-affinity receptors		
β_c	K_d, nM	Receptors per cell, no. × 10^{-3}	K_d, pM	Receptors per cell, no. × 10^{-3}	*P*
None	5.9	210			NS
	3.7	230			NS
Wild type	1.8	62	5	2.3	0.001
	2.1	120	52	6.4	0.004
KMR362-4GGG	1.3	73	17	9.2	<0.001
	2.1	130	28	5.4	<0.001
K362Q	1.8	66	15	2.9	<0.001
	2.6	130	22	7.7	0.008
K362E	1.9	44	120	7.9	0.03
	2.3	110	94	1.4	0.004
R364K	2.2	32	76	9.4	0.03
	0.9	85	16	5.8	<0.001
R364Q	3.5	73	34	4.4	<0.001
	1.7	98	52	14	0.002
R364E	1.3	62	17	4.2	<0.001
	1.9	110	40	10	0.002
Y365F	1.3	62	12	2.1	0.03
	2.4	69	35	1.9	<0.001
E366R	1.2	46	17	4.6	<0.001
	2.4	62	4	1.5	0.001
H367E	1.2	64	45	2.1	0.04
	2.1	52			NS
H367K	1.1	69			NS
	3.3	79			NS
H367Q	1.3	85			NS
	2.5	58			NS

Data are from two experiments in which COS cells were cotransfected with expression vectors encoding hGM-CSFRα and the wild-type or mutant forms of β_c indicated. NS, not significant.

critical binding determinant or, alternatively, by disturbing translation, folding, or transport of the receptor to the cell surface.

Where the β_c mutants could confer high-affinity binding, the estimated K_d values for high-affinity binding were generally within the range determined for cells expressing wild-type β_c (i.e., K_d = 5–52 pM) (Table 1). However, cells expressing the β_c mutant K362E (containing the Lys-362 → Glu substitution) exhibited K_d values of 120 and 94 pM in the two experiments, raising the possibility that this substitution slightly modifies high-affinity binding.

Most amino acid substitutions within β_c did not appear to diminish the expression level of mutant β_c protein. As shown in Table 1, comparable or greater numbers of high-affinity receptors were detected on cells transfected with the β_c mutant cDNAs ($1.4–14 \times 10^3$ receptors per cell) versus those transfected with wild-type β_c cDNA ($2.3–6.4 \times 10^3$ receptors per cell). The relative number of high-affinity receptors expressed on cells transfected with a given β_c mutant cDNA varied up to 6-fold in the two experiments (for example, see Table 1; β_c mutant K362E), and this result may have been from variation in transfection efficiency. Despite this variation, as few as $1.4–1.5 \times 10^3$ high-affinity receptors per cell could be readily detected (Table 1, see mutants K362E and E366R).

Cell-Surface Expression of β_c Mutants H367E, H367K, and H367Q. To determine whether the absence of high-affinity receptors on COS cells cotransfected with cDNAs encoding mutants H367E, H367K, and H367Q and GM-CSFRα coincided with a lack of cell-surface expression of the mutant β_c chains, transfectants were stained with a rat monoclonal antibody against β_c, CRS-1 (31), and fluorescein isothiocyanate-conjugated rabbit anti-rat serum, and the transfectants were detected by indirect immunofluorescence microscopy (Fig. 3). Cells transfected with GM-CSFRα cDNA alone lacked specific staining (Fig. 3*A*), whereas a different subset

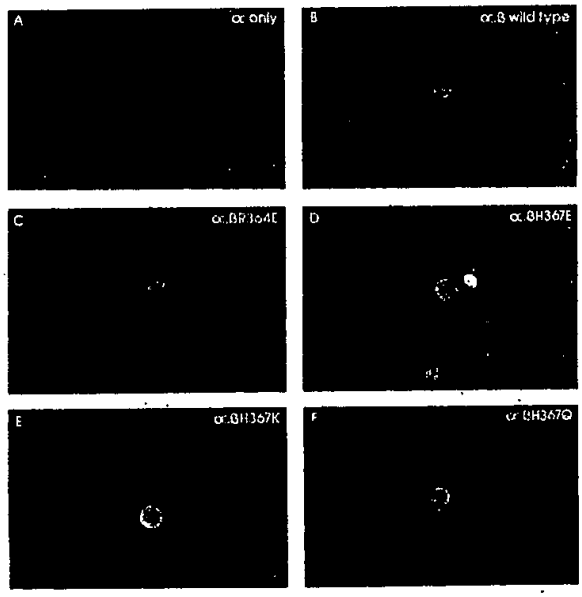

FIG. 3. Cell-surface expression of mutant β_c chains on COS cell transfectants. Cells were transfected with cDNAs for GM-CSFRα (*A*), GM-CSFRα and β_c wild type (*B*), or GM-CSFRα and the β_c mutants R364E (*C*), H367E (*D*), H367K (*E*), or H367Q (*F*). After 72 hr, cell-surface expression was detected by indirect immunofluorescent staining of cells with CRS-1, a monoclonal antibody against β_c (31) and fluorescein isothiocyanate-conjugated rabbit anti-rat antiserum.

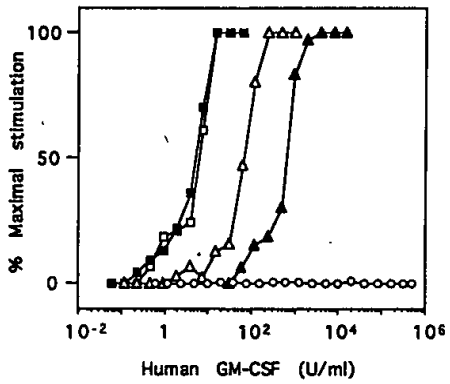

FIG. 4. Proliferative responses of stable CTLL-2 transfectants to hGM-CSF. Duplicate samples of ≈200 CTLLα1 (GM-CSFRα only) (○), CTLLαβ.3 (GM-CSFRα and β_c wild type) (□), CTLLαβ.4 (GM-CSFRα and β_c wild type) (■), CTLLαβ10.1 (GM-CSFRα and β_c H367K) (▲), and CTLLαβ10.2 cells (GM-CSFRα and β_c H367K) (△) were incubated in the presence of various concentrations of hGM-CSF for 48 hr. Maximal stimulation was defined as an average of ≥200 cells per well..

of cells (dependent on transfection efficiency) transfected with the GM-CSFRα and the wild-type β_c cDNAs clearly exhibited surface staining consistent with cell-surface expression of β_c (Fig. 3*B*). Identical patterns of staining were detected on cells transfected with cDNAs for mutant R364E (Fig. 3*C*), which exhibited affinity-converting activity similar to wild-type β_c (Table 1) and the mutants H367E, H367K, and H367Q, which did not confer high-affinity binding (Fig. 3 *D–F*). Indeed, all β_c mutants were expressed at the cell surface by this criterion. These results clearly indicate that His-367 → Glu, His-367 → Lys, and His-367 → Gln substitutions did not prevent cell-surface expression of these mutant β_c proteins.

Biological Activity of β_c Mutant H367K. The ability of β_c H367K mutant to deliver a proliferative signal was tested by installing GM-CSFRα and β_c wild type or β_c H367K in the murine IL-2-dependent cell line CTLL-2 and determining responsiveness of these cells to hGM-CSF. Fig. 4 shows that ≈5 units of hGM-CSF per ml was required to stimulate half-maximal proliferation of two cell lines, CTLL$\alpha\beta$.3 and CTLL$\alpha\beta$.4, transfected with cDNAs for GM-CSFRα and wild-type β_c, whereas parental CTLLα1 cells expressing GM-CSFRα alone did not respond to hGM-CSF. Similar results have been reported (4) and are consistent with a requirement for both receptor subunits to initiate proliferation. By contrast, two cell lines transfected with cDNAs encoding GM-CSFRα and β_c mutant H367K, designated CTLL$\alpha\beta$10.1 and CTLL$\alpha\beta$10.2, required hGM-CSF at 600 units/ml or 60 units/ml, respectively, to achieve half-maximal stimulation. The reduced responsiveness of these cells to hGM-CSF is consistent with the observed loss of affinity-converting activity of β_c H367K in COS cells. The responsiveness of transfected and untransfected CTLL-2 cells to IL-2 was similar (data not shown), indicating that transfection did not affect the proliferative potential of individual cell lines.

DISCUSSION

The identification of Glu-21 of hGM-CSF as a residue critical for binding to the high-affinity GM-CSF receptor but not to the GM-CSFRα (18, 19), together with the observation that human β_c is a component of the high-affinity GM-CSF receptor but lacks intrinsic GM-CSF-binding activity (6), implies that GM-CSF interacts directly with β_c during for-

mation of the high-affinity receptor complex only after binding to GM-CSFRα has occurred. To locate residues in β_c involved in this interaction we first identified a region encompassing Lys-362–His-367 in the second hemopoietin domain of β_c, which corresponds in position to a major ligand-binding determinant in the loop between the B and C strands of hGHR (16) and also colocalizes with likely cytokine-binding sites in murine β_{IL-3} (24) and human IL-2 receptor β subunit (23). Several mutant β_c chains with amino acid substitutions in this region were generated by site-directed mutagenesis, and their capacity to contribute to high-affinity binding and signaling when coexpressed with GM-CSFRα was assessed.

Substitution of individual amino acids in β_c did not appear to disrupt the synthesis, processing, transport, conformation, or functional activity of β_c. Both wild-type and mutant forms of β_c were clearly expressed on the surface of COS cell transfectants stained indirectly with CRS-1, a monoclonal antibody against β_c (31). Moreover, the β_c mutants KMR362-4GGG, K362Q, R364K, R364Q, R364E, Y365F, and E366R were similar to wild-type β_c in their capacity to confer high-affinity binding, indicating that these proteins were correctly folded. Furthermore, β_c H367K mutant, which lacked detectable affinity-converting activity when expressed on COS cells, conveyed a proliferative signal when coexpressed with GM-CSFRα in CTLL-2 cells, although at higher concentrations of hGM-CSF than were required to stimulate proliferation of cells expressing GM-CSFRα and β_c wild type, suggesting that this β_c mutant was structurally intact.

The diminished responsiveness to hGM-CSF of CTLL-$\alpha\beta$10.1 and CTLL$\alpha\beta$10.2 lines, which express GM-CSFRα and β_c H367K, compared with CTLL$\alpha\beta$.3 and CTLL$\alpha\beta$.4 lines, which express GM-CSFRα and β_c wild type, presumably reflects the loss of affinity-converting activity of β_c mutant H367K. The capacity of these cell lines to respond to hGM-CSF resembles the ability of FDC-P1 or CTLL-2 cell lines expressing human GM-CSFRα and either endogenous or exogenous AIC2B protein, respectively, to proliferate in the absence of high-affinity binding (4, 12). Presumably β_c mutant H367K, like AIC2B protein, can form a functional signal-transduction complex with human GM-CSFRα but with a binding affinity too low to detect.

Because all β_c mutants could be detected at the cell surface, the modest reduction in the affinity of GM-CSF binding seen for the K362E mutant and the failure of H367K and H367Q mutants to contribute to high-affinity binding are most likely due to disruption of the GM-CSF-binding site. However, whether the residues Lys-362 and His-367 directly contact GM-CSF or whether they are indirectly required to maintain the local structure of the binding site remains unclear. One way to test whether mutant H367 interacts directly with Glu-21 of hGM-CSF would be to determine whether complementary mutations in Glu-21 of hGM-CSF could restore the high-affinity binding capacity of β_c H367 mutants. Recent studies with human IL-2 receptor β chain revealed that point mutations within the sequence, Ser-132–His-133–Tyr-134 (SHY), which is located in the analogous region to Lys-362–His-367 of β_c, abolished binding of murine IL-2 but enhanced the affinity of human IL-2 receptor β subunit for variants of murine IL-2 with amino acid substitutions of Asp-34 (23). Interestingly, Asp-34 in the N-terminal helix of murine IL-2 is in an analogous position to Glu-21 of hGM-CSF, and it is therefore tempting to predict that the loss of high-affinity binding associated with the H367K and H367Q mutations in β_c might be complemented by amino acid substitutions involving Glu-21 of hGM-CSF.

Despite the apparently critical role of His-367 of β_c in high-affinity binding of GM-CSF, additional residues located in different regions of the molecule probably participate in ligand binding. For comparison, the interface between subunit 2 of the hGHR and site 2 on hGH, involving an analogous interaction to that between β_c and GM-CSF, requires four residues within hGHR and three residues in hGH for the intermolecular hydrogen and ionic bonds alone and several others for the hydrophobic interactions (16).

An issue still to be addressed is whether disruption of the GM-CSF binding site in β_c affects binding of IL-3 or IL-5 to their respective high-affinity receptors, which also use β_c. Like hGM-CSF, human IL-3 and human IL-5 also possess an acidic residue in the analogous position to Glu-21, and it will be interesting to determine whether this residue contributes to the binding site that enables all three of these cytokines to interact with β_c.

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